TATUMINE, A PEPTIDE FROM *BACILLUS BREVIS* Vm4-572-403

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The mutant 572-403 of *Bacillus brevis* Vm4 was isolated from *N*-methyl-*N'*-nitro-*N*-nitroso guanidine (nitrosoguanidine)-treated cultures of the parental strain. The mutant produces tatumine and edeine B when grown in a defined medium at 40°C. Tatumine is composed of spermidine, glycine, isoserine, and an unidentified ninhydrin-positive compound; it has a molecular weight of less than 730 d (edeine A). It is soluble in water and isopropanol. Tatumine has no antimicrobial activity but has selective cytotoxicity against cultured cells of sarcoma origin. This activity is not destroyed by heating for 1 hour at 60°C.

In search of mutants of *Bacillus brevis*[1,2] which would produce peptide antibiotics other than edeine A and B[3,4], we have isolated a mutant which produces in addition to edeine B a novel antibiotic tatumine[5]. This report describes the method of isolation of tatumine, its partial chemical composition, and chemical and biological properties.

Materials and Methods

Bacillus brevis Vm4 was grown to log phase at 37°C in a rich medium. Mutants were produced by treatment with nitrosoguanidine[6]. Strain 572-403 was isolated from colonies which were replicated on plates with a solid defined medium and incubated for 24 hours at 40°C. The defined medium consisted of 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.2 mg $MgSO_4 \cdot 7H_2O$, 20 mg $MnSO_4$, 10 mg $FeSO_4$, 20 mg $CaCl_2$, 2.5 g glycine, 2.5 g L-glutamine, 0.6 g L-asparagine, 0.8 g D,L-methionine, and 20 mg thiamine, per liter; the pH was adjusted to 7.2. To label components of tatumine and edeine B the following radiochemicals were added to this medium: 2 μCi/ml of (terminal methylene-^{3}H)-spermidine·3HCl (specific activity 15 Ci/mmol), or (U-^{14}C)-glycine, or (1,4-^{14}C)-spermidine (each of specific activity 100 Ci/mol), or L-(U-^{14}C)-tyrosine (specific activity 450 Ci/mol). The ^{14}C-radiochemicals were purchased from Amersham Corporation, Arlington Heights, IL, the ^{3}H-radiochemicals from New England Nuclear, Boston, MA (U.S.A.).

To prepare tatumine, strain 572-403 was grown in the defined medium at 40°C for 16 hours with vigorous shaking. Tatumine was isolated from the fluid of 2-liter cultures after the bacterial debris was removed by centrifugation. The supernatant was filtered (0.45 μm pore-size filter (Nalge Sybron Corp., Rochester, NY, U.S.A.)) and applied to a column containing 100 g of Dowex 50×8 (H^+ form). Two liters of distilled water were passed through the column followed by 1 liter of 1 N NH_4OH. The fractions which eluted at pH 10.2～11.2 were pooled, concentrated *in vacuo*, and mixed with 10 g of CM-32 (carboxymethyl cellulose (Whatman Ltd., Maidstone, Kent, UK)). The cellulose was dried *in vacuo* and packed into a column (2.4×11 cm), through which isopropanol (500 ml) and isopropanol-NH_4OH (28～30% as NH_3) (80: 3, v/v) solutions were then passed. Fractions of 2.5 ml were collected and tatumine was eluted by the second solution in fractions 5 through 10. These fractions were pooled, concentrated *in vacuo*, and applied to a column of Sephadex G-15 (1×150 cm) (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.1 M NH_4HCO_3. Tatumine was eluted as a single peak midway between edeine B and spermidine standards; the peak fractions were taken to dryness and stored at

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$-20°C$. Tatumine was identified as a ninhydrin-positive spot by high voltage paper electrophoresis under the conditions described in Table 2.

The toxicity of tatumine to cells in culture was assessed by incubating 2×10^5 cells in 0.25 ml total volume medium (in disposable polyvinyl plates) in the presence of $10^{-8}\sim10^{-6}$ M tatumine (based on an estimated molecular weight of 400 daltons) and 0.5 μCi of L-4,5-^{3}H (N)-leucine (specific activity 5 Ci/mmol), or (methyl-^{3}H)-thymidine (specific activity 2 Ci/mmol) for 24 hours in a 5% CO_2-humidified incubator. The cultures were photographed before and after treatment. Cold 5% trichloroacetic acid (TCA) - 0.3 M $Na_2P_2O_7$ (0.4 ml) was added and the precipitate was collected by centrifugation, washed 6 times with cold 5% TCA, and 3 times with cold absolute ethanol. The radioactivity of the precipitated material was determined as described previously[4]. The incorporation of radiolabeled leucine or thymidine into TCA-precipitable material by each tatumine-treated culture was expressed as a percentage of its control culture value. A limited survey of cells in culture was made using human osteosarcoma (0327), rhabdomyosarcoma (RD 114), melanoma (D and G), cervical carcinoma (HeLa), Burkitt lymphoma (ESP-1), normal rat kidney (NRK 2) and, mouse sarcoma (XC). Stocks of cells and some of the assays were kindly provided by Dr. J. A. GEORGIADES (Department of Virology, M.D. Anderson Hospital and Tumor Institute, University of Texas System Cancer Center, Houston, TX, U.S.A.).

The antimicrobial activity of tatumine was tested in the same manner as described for edeine A[4].

The amino acid analysis was performed on a TSM automated amino acid analyzer (Technicon Corporation) using standard buffers. Tatumine and edeine A or B[7] were completely hydrolyzed in 6 N HCl, by 24 hours, at 100°C, *in vacuo*.

Results

Tatumine was isolated from the fermentation broth of *Bacillus brevis* Vm4-572-403 by adsorption to Dowex 50×8 (H^+ form) and elution with 1 N NH_4OH at pH $10.2\sim11.2$. It was purified by chromatography on a carboxymethyl cellulose column with isopropanol - NH_4OH ($28\sim30$% as NH_3) (80: 3, v/v) as the eluent. Tatumine, which eluted as a single, discrete peak from the column containing Sephadex G-15, was shown to be a single, ninhydrin-positive component under the conditions of paper high voltage electrophoresis and chromatography listed in Table 1. Areas corresponding to tatumine were eluted with water from paper. Tatumine was subjected to paper electrophoresis and chromatography (see Table 1) in different sequences without any change in the location of the ninhydrin-positive spot or in cytotoxicity; this indicates that tatumine was homogeneous. Radiolabeled tatumine was also subjected to preparative paper high voltage electrophoresis under the conditions described in Table 2 and it co-migrated with the non-radioactive preparation.

The composition of tatumine was examined after acid hydrolysis in 6 N HCl, at 100°C, for 24 hours, *in vacuo*. Four ninhydrin-positive components were detected in the hydrolysates by paper chromatography, or, paper high-voltage electrophoresis, or, amino acid analysis. When cultures of strain 572-403 were supplemented with ^{14}C-glycine, ^{3}H-spermidine, and ^{14}C-tyrosine, tatumine hydrolysates contained ^{14}C-glycine and ^{3}H-spermidine, but not ^{14}C-tyrosine, while edeine B hydrolysates had all three of these radiochemicals (Table 2). In tatumine hydrolysates, an additional non-radioactive, ninhydrin-positive, component was detected by high voltage paper electrophoresis (2.8 cm from origin migrating towards cathode), and, by amino acid analysis (eluting at 150 minutes) (Table 2); hydrolysis of edeine A or B produces isoserine which has these same mobilities[7]. Tatumine hydrolysates also contained a relatively less ninhydrin-positive, non-radioactive component which was evident by paper high-voltage electrophoresis (21.3 cm from origin) and by amino acid analysis (eluting at 180 minutes); no component of edeine A or B has identical mobilities.

Table 1. Characteristics of tatumine and edeine A.

Property	Edeine A	Tatumine
Chemical form	Peptide	Peptide
Composition	Spermidine, glycine, 2,3-diamino-propionic acid, 2,6-diamino-7-hydroxyazelaic acid, isoserine, isotyrosine	Spermidine, glycine, isoserine, unidentified ninhydrin-positive component
Initial ninhydrin color*	Bluish-purple	Light purple
Molecular weight	730	>335
Absorption maximum (nm)	272	206
Solubility	Water	Water and isopropanol
Paper chromatography (Rf)		
Butanol - H_2O - CH_3COOH (4: 1: 5)	0.10	0.76
Ethanol - H_2O - NH_4OH (18: 1: 1)	0.05	0.80
Paper high voltage electrophoresis (cm from origin)**	25	18

* On chromatograms developed at room temperature after dipping into 0.25% ninhydrin in acetone.
** Electrophoretic conditions are described in Table 2.

Table 2. High-voltage electrophoresis and amino acid analysis of tatumine and edeine A after hydrolysis in 6 N HCl at 100°C for 24 hours.

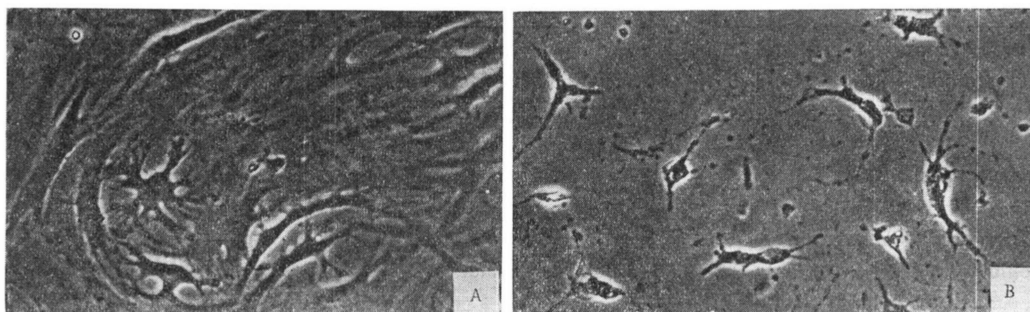
Residue	High-voltage electrophoresis* Distance of migration from origin (cm)		Amino acid analysis** Time of elution (min) Column I	
	Tatumine	Edeine A	Tatumine	Edeine A
Glycine	2.8***	2.8***	120	120
Isoserine	4.0	4.0	150	150
2,6-Diamino-7-hydroxyazelaic acid	absent	11.5	absent	320
Unidentified	21.5	absent	180	absent
			Column II	
Isotyrosine	absent	9.8***	absent	30
2,3-Diamino-propionic acid	absent	27.5	absent	70
Spermidine	48.0***	47.8***	—	—

* Electrophoresis was carried out on paper (3MM, Whatman Ltd. UK) in pyridine-acetic acid-water (10 : 100 : 890), pH 3.5, at 4°C, 1.5 hours, 45 V/cm.
** Chromatographic column I: 55 cm, elution with buffer of pH 3.25 for 140 minutes followed by buffer of pH 4.25. Column II: 12 cm, elution with buffer of pH 5.28.
*** This indicates the position of radioactivity in hydrolysates of tatumine and edeine A obtained from cultures which were grown in the presence of radiolabeled glycine, spermidine, and tyrosine (see text).

A comparison of the properties of edeine A and tatumine is given in Table 1. Tatumine reacts with ninhydrin to give a light pink color, while edeine A gives a blue-purple color. Tatumine has an ultraviolet absorbance maximum at 206 nm, suggesting it is a peptide. In contrast to edeine A and B, it has no absorbance maximum near 272 nm, indicating an absence of aromatic amino acid residues. Tatumine is soluble in water and isopropanol. Upon paper chromatography, tatumine has a high mobility in acidic and basic solvent systems. Tatumine, like edeine A or B, contains neither sulfur (or sulfate) nor phosphorus (or phosphate (results not shown)).

Tatumine was shown to have a molecular weight less than edeine A (730 daltons) but greater

Fig. 1. Cell culture of human osteosarcoma 0327 after 24-hour exposure to tatumine (10^{-7} M). Culture without tatumine (A). Culture with tatumine (B). Phase contrast 160 ×.



than spermidine by molecular sieve column chromatography (Sephadex G-15), and, by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea of ^{14}C-spermidine-containing components (results not shown). The three identified ninhydrin-positive components of tatumine have a total molecular weight of 335 daltons. Tatumine, like edeine A and B, is resistant to digestion with common proteolytic enzymes. It is also resistant to carboxypeptidase B, the enzyme which cleaves edeine A or B into two peptides which are biologically inactive[8)].

Tatumine affects the growth of cultured cells derived from human and animal sarcoma. After 24 hours of incubation with $10^{-6}\sim10^{-8}$ M tatumine, 95% of the cells of human sarcoma type 0327 (see Fig. 1), RD 114, and animal sarcoma type XC, were detached from the culture plates. They were of ragged, irregular morphology, while the control cells were flattened, of regular morphology, and nearly confluent. At the concentrations of tatumine noted above, the incorporation of radioactivity into TCA-precipitable material of precursors of protein and DNA was less than 5% of the controls at 24 hours of incubation (data not shown). When tested at the highest concentration (10^{-6} M), the tatumine-resistant cells, melanoma (D and G), HeLa, NRK-2, and ESP-1, were of unchanged morphology and incorporated the radioactive precursors of protein and DNA almost as readily (>90%) as control cultures. The cytotoxic effect of tatumine against cultured cells of sarcoma origin is not destroyed by heating of tatumine at 60°C for 1 hour.

In contrast to edeine A and B, which are wide-spectrum antibiotics, tatumine showed no antimicrobial activity.

Tatumine is a peptide which contains three components of edeine A, namely, spermidine, glycine, and isoserine, and, in addition, one other, unidentified, ninhydrin-positive component. The sequence of edeine A is: *N*-isotyrosyl-isoseryl-(α,α)-2,3-diaminopropionyl-(α,α)-2,6-diamino-7-hydroxyazelayl-(ω,α)-glycyl-spermidine[8)]. If tatumine has a similar sequence, it resembles one end of the edeine A molecule, but the next two edeine A amino acids are deleted before isoserine is attached. It is worth pointing out that while tatumine has cytotoxic activity against several types of sarcoma cells in cultures, the 2,3-diaminopropionyl-2,6-diamino-7-hydroxyazelayl-glycyl-spermidine fragment of edeine A does not.

Further studies of the structure of tatumine are in progress.

Acknowledgments

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